

Investigation of *Bacillus pumilus* and *Staphylococcus haemolyticus* adhesion on 304L Stainless Steel in Atmospheric Simulated Medium

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Abstract

The bacteria adhesion on materials surface is the first step to formation of biofilm that could lead to biocorrosion. This study aimed to investigate the correlation between bacteria adhesion and the physicochemical properties of both materials and bacteria in atmospheric simulated medium. The material used in this study is 304L stainless steel and *Bacillus pumilus* and *Staphylococcus haemolyticus* bacteria strains. The hydrophobicity, electron donor and electron receptor properties were determined by contact angle measurements. In addition, the bacteria capacity of adhesion was followed by using a scanning electron microscope. The contact angle measurements finding showed that both bacteria have hydrophilic surfaces qualitatively and quantitatively. Otherwise, the stainless steel has revealed a hydrophobic character qualitatively and quantitatively. Moreover, the scanning electron microscope displayed the high speed of adhesion of the studied bacteria. The contact angle measurements could be used as a mean to prevent biocorrosion caused by bacteria adhesion.

Keywords: Adhesion, stainless steel, atmospheric simulated medium, biocorrosion

Introduction

Microbiologically Influenced Corrosion (MIC) or biocorrosion is an electrochemical phenomenon in which microorganisms adhere to the surfaces of metals or other materials. This adhesion can induce or accelerate corrosion reactions of these materials through the interfacial interaction with the metabolic activities of these microorganisms (Javaherdashti, 2016). This process of biocorrosion could be able to initiate, facilitate or accelerate

corrosion reactions through the interaction of the three components: metal, solution and micro-organisms (De Romero et al., 2004).

It is, in fact, interactions between the living world and materials via adhesion; any material in contact with a biologically active medium is likely to be a victim of biocorrosion. Actually, the microorganisms don't "nibble" the material, but they change, drastically, due to their metabolism, the physical chemistry environment of the material interface (pH, oxygen concentration, chemical concentration, ...) creating the conditions triggering the biocorrosion (Videla, 2002; Videla & Herrera, 2004) . Microorganisms can be considered as great catalysts of the phenomenon of electrochemical nature: corrosion.

The adhesion of microorganisms to surfaces is the first step in biofilm formation (Queiroz et al., 2018). It depends on the interaction energy between the surface in the presence of microorganisms, and the surrounding environment (Katsikogianni & Missirlis, 2004). Thus, many parameters may alter these interactions can potentially influence microbial adhesion to a solid support.

Therefore, the use of the contact angle goniometer, which is an analysis method of surfaces, has been implemented in order to describe the physicochemical properties of microorganisms surface (Sadiki et al., 2017) (*Bacillus pumilus* and *Staphylococcus haemolyticus* in this case, which were isolated from a 304L stainless steel plate) in term of hydrophobicity and electron acceptor / donor properties.

Subsequently, it was interesting to evaluate the adhesive behavior of these microorganisms on the surface of the 304L stainless steel using the Environmental Scanning Electron Microscopy (ESEM).

Most of biocorrosion studies have conducted their researches in acidic medium (Agarry et al., 2018) or seawater simulated medium (Rasheed et al., 2018) or cooling tower water solution (Narenkumar et al., 2019), that why this research has the privilege to be the first one that uses a simulated atmospheric medium to study the bacteria adhesion on a metallic surface.

The aim of this work was to investigate the correlation between adhesion and the physicochemical properties of 304L stainless steel surface and bacteria surface (*Bacillus pumilus* and *Staphylococcus haemolyticus*) in atmospheric simulated medium.

Materials and Methods

1) Bacteria isolation, growth conditions and identification

Bacillus pumilus and *Staphylococcus haemolyticus* were isolated from a 304L stainless steel plate using moist sterile cotton-tipped swabs. The swabs are then used to transfer bacteria to petri dishes filled with Lysogeny broth (LB) agar medium (5g L⁻¹ yeast extract, 10g L⁻¹ Tryptone, 10g L⁻¹ NaCl, 20g

L⁻¹ agar) prepared with atmospheric simulated water, which were incubated in 37°C for 24 hours.

Succeeding the work of Rocca et al. (2004), the international standard ASTM D1384 solution was used to simulate atmospheric corrosion (148 mg L⁻¹ Na₂SO₄, 138 mg L⁻¹ NaHCO₃ and 165 mg L⁻¹ NaCl). The molecular identification of the strains is conducted by PCR-sequencing process. This method consists in carrying out a succession of replication reactions of double stranded DNA template. After extracting the genomic DNA of the various isolates, amplification of the 16S rDNA for bacteria by PCR was performed. Primers that were used are universal primers: FD1 and RS16 primers were used to identify bacteria (Mostakim et al., 2011). The resulting PCR product was sequenced by the automated technique of Sanger using the ABI 3130 (Applied Biosystems, France) sequencing machine. Finally, the BLAST (Basic Local Alignment Search) data base tool was used for sequences identification.

2) Substrate preparation

The stainless steel specimens were purchased from Sonasid Company (Nador-Morocco). The nominal elemental composition (wt %) of the 304L sample was: Fe 71.376%, Ni 8.18%, C 0.053%, Cr 18.08%, Cu 0.06%, Mn 1.68%, Mo 0.05%, N 0.047%, P 0.037%, S 0.007%, and Si 0.43%. Rectangle-shaped specimens with dimensions of 10 mm x 25 mm and thickness of 3 mm were used for SEM and contact angle analysis. The specimens were cut from the original plate samples with the dimension of 100 x 100 x 3 mm. Prior to the experiments, each specimen was sequentially ground with a series of emery papers (of 180, 600, 800, and 1200 grade) to a smooth surface, rinsed with sterile deionized water thrice, degreased in acetone, followed by sterilizing in 70% ethanol for 8 h, and then dried aseptically in a laminar flow cabinet. The newly prepared specimens were immediately immersed in the test medium for all of the experiments.

3) Determination of the physicochemical properties of bacteria and stainless steel surfaces - Contact angle measurements

Physicochemical properties of the microbial cell and stainless steel surfaces were determined using a goniometer apparatus by the sessile drop method (Hassan et al., 2014; Barkai et al., 2015; Chen 2015). The bacteria was suspended in KNO₃ (0.1M) sterile solution, followed by centrifugation at 10.000 g for 15 min. The pellet was then washed twice with sterile KNO₃ and re-suspended in the same solution at a concentration of 10⁸CFU/ml. were filtered throughout a cellulose acetate membrane filter (0.45µm) by using a negative pressure system. For each strain, three independently grown cultures were used, from which three filters of each were prepared and measured. For the determination of interfacial free energy of the solid surface (bacteria and

stainless steel), three liquids are recommended (Van Oss, 1993). They consist of two polar liquids (Water and Formamide) and one apolar liquid (Diiodomethane) with known surface tension characteristics (Table 1). The initial contact angle of each liquid was measured after drop stabilization on the solid sample surfaces. Three measurements of contact angles were made on each surface of substrate for all probes.

Table 1: Surface energy properties of pure liquid used to measure contact angles

Liquid	$\gamma^{LW}(\text{mJ/m}^2)$	$\gamma^+(\text{mJ/m}^2)$	$\gamma^-(\text{mJ/m}^2)$
Water (H ₂ O)	21.8	25.5	25.5
Formamide (CH ₃ NO)	39	2.3	39.6
Diiodomethane(CH ₂ I ₂)	50.5	0.0	0.0

4) Hydrophobicity and surface free energy

The surface physicochemical properties, including hydrophobicity and surface free energy of bacteria were determined through the contact angle measurements using a goniometer (GBX Instruments) and the calculations using the Van Oss (1993) approach.

According to Vogler (1998), a surface is hydrophobic quantitatively if the measurement of θ_w is greater than 65°, and it is considered quantitatively hydrophilic if θ_w is less than 65°.

In addition, conferring Van Oss (1993) approach, the degree of hydrophobicity of a given material is expressed as the free energy of interaction between two entities of that material when immersed in water (w): ΔG_{wi} . If the interaction between the two entities is stronger than the interaction of each entity with water, the material is considered qualitatively hydrophobic ($\Delta G_{wi} < 0$); conversely, for a qualitatively hydrophilic material ($\Delta G_{wi} > 0$). ΔG_{wi} is calculated through the surface tension components of the interacting entities, according to the following formula:

$$\Delta G_{wi} = -2\gamma_{iw} = -2\gamma_i [((\gamma_i^{LW})^{1/2} - (\gamma_w^{LW})^{1/2})^2 + 2((\gamma_i^+ \gamma_i^-)^{1/2} + (\gamma_w^+ \gamma_w^-)^{1/2} - (\gamma_i^+ \gamma_w^-)^{1/2} - (\gamma_w^+ \gamma_i^-)^{1/2})]$$

where γ^{LW} accounts for the Lifshitz–van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively.

The surface tension components of a solid material are obtained by measuring the contact angles of three pure liquids (one apolar and two polar) with well-known surface tension components (Absolom et al., 1983), followed by the simultaneous resolution of three equations of the following form:

$$\gamma_L (\cos\theta + 1) = 2 [(\gamma_s^{LW} \gamma_L^{LW})^{1/2} + (\gamma_s^+ \gamma_L^-)^{1/2} + (\gamma_s^- \gamma_L^+)^{1/2}]$$

5) Adhesion assay

The adhesion of investigated bacterial strains to the stainless steel surfaces was performed by sedimentation (Yuan et al., 2008). The stainless steel coupons, prepared such as previously mentioned, were immersed in bacterial suspension concentrated at 10^9 CFU/ml. The coupons were thus brought into contact with the bacterial cell surfaces for 2, 4 and 6 hours at 25 °C. After incubation, the surfaces were rinsed vigorously with sterile distilled water to remove non-adherent bacteria (Herald & Zottola, 1988; Briandet et al., 1999).

6) Environmental scanning electron microscopy analysis

After the adhesion of the two isolates on the stainless steels, the specimens were imaged using Environmental Scanning Electronic Microscopy (ESEM) Quanta 200. This device is equipped with tungsten filament (FEI Company).

7) Statistical analysis

All data were subjected to one-way ANOVA test, performed with the software package statgraphics Centurion XIV. Differences were considered significant at the $p < 0.05$ level of probability.

Results

1) Molecular identification of microorganisms

The analysis of the obtained sequences of the 16S rRNA gene and the comparison with the Gen-Bank BLASTN database allowed the molecular identification of the two isolates. The results indicated that the amplified sequences of the two bacteria exhibited a 99 % homology percentage to the 16S rRNA gene of *Bacillus pumilus* and *Staphylococcus haemolyticus*.

2) Physicochemical properties of bacteria

The physicochemical properties of bacteria surface presented in Table 2 revealed that both the bacteria have hydrophilic surfaces qualitatively and qualitatively with $\theta_w = 55,9^\circ$, $\Delta G_{\text{wi}} = 27,32 \text{ MJ.m}^{-2}$ for *Bacillus pumilus* and $\theta_w = 33,7^\circ$, $\Delta G_{\text{wi}} = 29,25 \text{ MJ.m}^{-2}$ for *Staphylococcus haemolyticus*. The isolate of the *Staphylococcus haemolyticus* showed the most hydrophilic character.

In addition, the results demonstrated that both studied strains were mainly electron acceptor with high values of $\gamma^+ = 23.5 \text{ mJ/m}^2$ for *Bacillus pumilus* and $\gamma^+ = 40.4 \text{ mJ/m}^2$ for *Staphylococcus haemolyticus*, and a small electron donor character $\gamma^- = 12.7 \text{ mJ/m}^2$ for *Bacillus pumilus* and $\gamma^- = 13.2 \text{ mJ/m}^2$ for *Staphylococcus haemolyticus*.

Table 2: Results of the hydrophobic properties and electron donor and acceptor character of the surface of isolates

Isolate	Contact angle ($^{\circ}\pm SD$) ^a			Surface tension (mJ m ⁻²)			$\Delta Giwi$
	$\theta_w(^{\circ})$	$\theta_F(^{\circ})$	$\theta_D(^{\circ})$	γ_{LW}	γ^+	γ^-	
<i>Bacillus pumilus</i>	55.9 $\pm 0,2$	46.6 $\pm 0,1$	97.9 $\pm 0,4$	9.5 $\pm 0,1$	23.5 $\pm 0,2$	12.7 $\pm 0,1$	27,32
<i>Staphylococcus haemolyticus</i>	33.7 $\pm 0,1$	29.7 $\pm 0,3$	91.4 $\pm 0,1$	12.1 $\pm 0,2$	40.4 $\pm 0,3$	13.2 $\pm 0,1$	29,25

^a \pm Standard deviations of three measures

3) Physicochemical properties of 304L stainless steel

The physicochemical characteristics of 304L stainless steel were presented on the Table 3.

Based on these results, stainless steel presented a hydrophobic character qualitatively and quantitatively ($\theta_w = 63,7$; $\Delta Giwi = -21,1$ MJ.m⁻²), with a weak character of electron donor and none electron acceptor character.

Table 3: Results of the hydrophobic properties and electron donor and acceptor character of the 304L stainless steel surface

Substrate	Contact angle ($^{\circ}\pm SD$) ^a			Surface tension (mJ m ⁻²)			$\Delta Giwi$
	$\theta_w(^{\circ})$	$\theta_F(^{\circ})$	$\theta_D(^{\circ})$	γ_{LW}	γ^+	γ^-	
304L stainless steel	63,7 $\pm 0,13$	50,0 $\pm 0,06$	36,1 $\pm 0,04$	41,4 $\pm 0,3$	0,0 $\pm 0,02$	18,7 $\pm 0,1$	-21,1

^a \pm Standard deviations of three measures

4) Adhesion assay of bacteria on 304L stainless steel surface's

The analysis of the surfaces of the stainless steel coupons by the ESEM shows that both the two isolates are able to adhere to the mentioned surface. The ESEM images (Fig.1) showed the strong capacity of adhesion of both bacteria. The strain of *Staphylococcus haemolyticus* (Fig.1-E, F and G) has displayed a bigger ability of adhesion than *Bacillus pumilus* (Fig.1-B, C and D). Both the strains have presented their adhesion ability within two hours' time. In addition, the micrographs displayed that the adhesion concentration increase with time, especially for *Staphylococcus haemolyticus* strain.

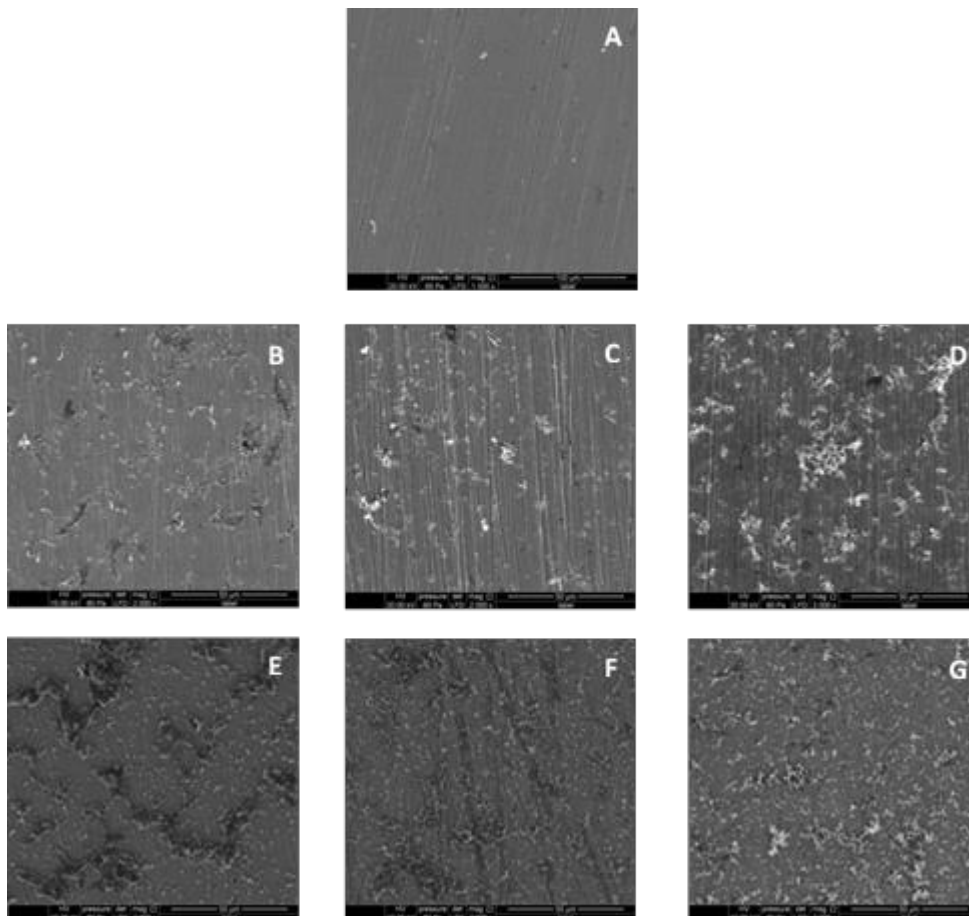


Fig.1. Electron micrographs of bacteria adhesion on 304L stainless steel surface

A: stainless steel surface without adhesion

B: Adhesion of *Bacillus pumilus* after 2H

C: Adhesion of *Bacillus pumilus* after 4H

D: Adhesion of *Bacillus pumilus* after 6H

E: Adhesion of *Staphylococcus haemolyticus* after 2H

F: Adhesion of *Staphylococcus haemolyticus* after 4H

G: Adhesion of *Staphylococcus haemolyticus* after 6H

Discussion

As generally known, hydrophobic bacteria adhered to a support which has the same character, as well as bacteria and a surface which are hydrophilic. As revealed in the Figure 1 and Tables 2 and 3, there is a contradiction between the theoretical predictions and the adhesion test results. The two isolates whose surface is hydrophilic have the ability to adhere to the surface of stainless steel which is hydrophobic, which indicates that hydrophobicity has no influence on the adhesion of bacteria to the surface of stainless steel, which is shown by El Abed et al. (2010) who reported that the

hydrophobic character does not influence *Aspergillus niger* membership and *Penicillium expansum* on the surface of cedar wood.

In addition, there are a numerous factors that can explain the contrariness between the theoretical predictions and the experimental observations. Bayouduh et al. (2006) reported that the theoretical prediction is limited to the first stages of attachment and does not account for the biological specific interactions. Some authors suggested other explanations and hypotheses that can be involved in the contrariness between theoretical predictions and experimental observations, for example, chemical and morphological heterogeneities of the membrane surfaces also some additional types of interactions including surface roughness and interactions between the membranes and the foulants (Molina et al., 1999; Grabbe & Horn, 1993; Yotsumoto & Yoon, 1993; Truesdail et al., 1998; Ducker et al., 1991).

Furthermore, the physico-chemical characteristics of a bacterial strain, including hydrophobicity and the electron donor / acceptor nature, are related to the composition of bacteria membrane. Boonaert & Rouxhet (2000) have reported that the hydrophilicity of two bacterial strains *Lactobacillus helveticus* and *Lactococcus lactis* is due to the high concentration of oxygen and a low concentration of hydrocarbons. Moreover, Hamadi et al. (2012) showed that the hydrophilicity of the cell surfaces correlates directly with the two functions (C (O, N)) and (OH, COC) which correspond to polysaccharides, and contrariwise with the function (C- (C, H)), which corresponds to hydrocarbons (Cuperus et al., 1993). The presence of oxygen and nitrogen to the surface of cells promotes interactions with water molecules (via hydrogen bonds) and therefore reduces its hydrophobicity.

The electrons donor / accept character of a bacterial strain is also related to the composition of the membrane, Hamadi et al. (2012) have shown that the phosphate group has the major role in defining the nature of electron donor (base), while the electron acceptor character is related to the low concentration of the amine group and the high concentration of polysaccharides.

Moreover, several works have reported that the hydrophobicity cannot always explain the results of microbial adhesion to the support (Pratt-Terpstra et al., 1988; Sjollem et al., 1990) and the acid-base interactions play a very important role in this phenomenon (Hamadi & Latrache, 2008; Henriques et al., 2004). Also, Van Oss (1993) reported that the acid-base interactions are 10-100 times more important compared to other interactions.

In addition, the stainless steel has revealed a hydrophobic character qualitatively and quantitatively with a weak character of electron donor and none electron acceptor character. This result correlates with the number of studies on the same type of stainless steel (304L), Rubio et al. (2002) reported

that the result of the measurement of the contact angle of 304L stainless steel surface showed that it is hydrophobic ($\theta_w = 78.9 \pm 3.2$), with a low electron donor character (6.4 mJ m⁻²) and almost no electron acceptor character (0.3 mJ m⁻²).

Conclusion

This work attempt to investigate *Bacillus pumilus* and *Staphylococcus haemolyticus* adhesion capacity on 304L stainless steel in atmospheric simulated medium. The adhesion test showed that both bacteria were able to adhere even though those results were not theoretically predicted. These results will be valuable for a better understanding of microbial adhesion that triggered biocorrosion.

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